Spring 2020

Identification of Saliva Using DNA Methylation Analysis for Forensic Use

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IDENTIFICATION OF SALIVA USING DNA METHYLATION ANALYSIS FOR FORENSIC USE

by

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A Thesis
Submitted to the Graduate School,
the College of Arts and Sciences
and the School of Criminal Justice, Forensic Science, and Security
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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May 2020

Published by the Graduate School

THE UNIVERSITY OF SOUTHERN MISSISSIPPI®

ABSTRACT

The identification of biological fluids is a precursor to determine if further human identification is required in a forensic setting. There are four forensically-relevant biological fluids: blood, semen, vaginal epithelial tissue, and saliva. While serological testing can identify these tissue types to some degree of accuracy, there has recently been momentum in research to use DNA methylation for tissue identification.

In the current study, five potential tissue-specific methylation markers were studied in order to identify locations in the genome that would differentiate saliva from other tissue types. Genomic DNA was extracted from each sample, followed by bisulfite modification, polymerase chain reaction amplification, and pyrosequencing. Pyrosequencing is a sequence by synthesis method that provides quantitative methylation data. The level of significance in methylation data between tissues was calculated using SPSS statistical package with a one-way ANOVA and Tukey's posthoc parameters. Two additional studies were completed: a species-specific test and mixture study.

Five loci, cg-9652652, cg-11536474, cg-3867465, cg-10781408, and cg-10122865 along with several adjacent CpG sites were found to be hypermethylated in saliva. The methylation data of saliva was statistically significant compared to other tissues, suggesting these markers can be used to discriminate saliva from other tissue types. In the species specificity study, it was observed that the primers used in one of the assays were human specific as they did not amplify non-primate samples. A mixture study using two different tissues behaved as predicted where a reduction in the methylation percent was observed when the quantity of salivary DNA decreased.

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my committee chair, Dr. Kuppareddi Balamurugan, along with committee members Dr. Dean Bertram and Dr. Xuyang He. Thank you for taking the time to review all my work and support me in the research phase of this process. I would also like to thank my friend and fellow graduate student Lauren Satcher, who also navigated this process with me and provided immense support. Finally, I would also like to express my thanks to faculty of The School of Criminal Justice, Forensic Science, and Security for their constant support during my academic career at The University of Southern Mississippi.

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CHAPTER I - INTRODUCTION

Forensic DNA Identification Methods

Within the last 35 years DNA technology has seen a steady and significant advancement, which has positively impacted the ability to identify individuals using biological evidence materials. In 1980 Ray White describes the first polymorphic VNTR marker; however, the use of DNA for human identification is credited to Alec Jeffreys, who developed a Restriction Fragment Length Polymorphism (RFLP) probe in 1985 that was able to identify variable number tandem repeat (VNTR) regions that are located within the human genome (Butler, 2010). VNTR regions, as the name implies, are genomic regions that contain a variable number of repeats between individuals; however, it is because the VNTR regions vary in both number of repeats and in the number of nucleotide bases per repeat that they are unique enough to distinguish between individuals. Jeffreys is the first to use the RFLP technique to find VNTRs for human identification purposes (Butler, 2010). While the RFLP probe was a huge milestone for forensic human identification, it was not without its disadvantages. VNTR analysis requires DNA that is non-degraded and in a quantity that is at least 100 nanograms (Hammond et al., 1994). Neither of these factors can be guaranteed of a DNA sample collected from a crime scene. The disadvantages of VNTR analysis prompted the search for a human identification technique that could overcome both the high quality and quantity barrier.

In 1983, a methodology was developed by Kary Mullis, which opened the door for the opportunity of a more efficient way of human identification (Saiki et al., 1985). This methodology was called polymerase chain reaction (PCR) and gave the ability to

amplify small quantities of DNA through successive cycles of systematic heating and cooling. PCR involves three main steps in each cycle: denaturation, annealing, and extension. The reaction is supplied with primers to bind single-stranded DNA, an excess of nucleotides, DNA polymerase enzymes to extend the DNA, and buffers ideal for these reactions to take place (Lynch & Brown, 1989). Each cycle effectively doubles the amount of DNA present in the sample in a relatively short time span; generally, amplification reactions take only a few hours. The ability to amplify DNA has allowed for a large advancement in many different branches of the scientific community. Forensically, the ability to amplify DNA gives more available sample for analysis.

Short Tandem Repeat (STR) markers contain a variable number of repeats at different locations in the genome that are unique to each individual. The current national DNA quality assurance standards call for the analysis of twenty STR markers for human identification. These markers are part of the Combined DNA Index System (CODIS), which is a DNA database maintained by the Federal Bureau of Investigation. The odds of two individuals, excluding identical twins, having the same number of repeats at the exact STR markers is virtually impossible. This is why STR markers have become the method of choice when comparing evidence from a crime scene to reference samples from the person(s) of interest. The ability to amplify DNA has allowed for the creation of commercial kits that can amplify specific STR segments of the human genome which are currently used for human identification (Romsos & Vallone, 2015).

Forensic Tissue Identification

While human identification has seen significant advancement in recent years, another aspect of forensic science, serological tests, have not seen similar progress.

Serological tests provide information pertaining to the identity of a biological fluid found at crime scenes. In identifying the source of the sample, the analyst can then decide what further testing may be required. Serological tests fall within two umbrella categories: presumptive tests and confirmatory tests. Presumptive tests are a quick means to determining what the sample identity could be, and a confirmatory test verifies the information gained from the presumptive test.

Currently there are several standard serological tests that are used to discriminate between forensic tissues of interest. Two of the most commonly used presumptive blood tests are Hemastix and the use of Phenolphthalein reagent. The presence is confirmed using a visual examination of ferroprotoporphyrin crystals that are produced in the presence of hemoglobin in the Takayama Crystal Test. Semen is presumptively identified using an Acid Phosphatase reagent, and confirmed using a visual observation of stained spermatozoa. There are also ABAcard tests for both blood and sperm, respectively, that confirm the presence of the substance in question using antigen-antibody interactions. Saliva is identified by the presence of an Amylase enzyme, which is found in higher quantity in saliva compared to all other biological samples.

While these tests do help in the discrimination of tissue types, they are not without fault. Currently there are no standard tests used to identify vaginal epithelial samples. Many of the tests listed above are also not specific to human biological samples, could result in false positives or negatives in cases where diluted samples are used, and the serological test reagents have the potential to degrade DNA (Gonçalves et al., 2017; Tobe et al., 2007; Vennemann et al., 2014). Some serological tests require a large amount sample, a luxury that is not always readily available in forensic casework. Also,

any sample that is used up for serological testing cannot then be used for identification purposes. This necessitates the need for a more reliable and streamlined protocol for forensic tissue identification.

DNA Methylation for Tissue Identification

Recently, there has been evidence to support DNA methylation analysis as a viable option for forensic tissue identification. DNA methylation is an epigenetic modification that occurs either naturally or in response to environmental stimuli such as age, smoking status, diet, and tissue type (Vidaki et al., 2013). In this process a methyl group is added to a cytosine base without changing the underlying sequence of the DNA itself (Li & Zhang, 2012). This type of modification occurs mostly in cytosine bases that are immediately followed by guanine bases. For this reason, the sites in the genome where this modification occurs are called CpG sites. Areas of the genome that contain a large amount of CpG sites in close proximity are called CpG islands (Vidaki, Daniel, & Court, 2013). Methylation levels have long been a topic of interest in the medical community, as the proximity of these CpG islands to the promoter region of genes can have an effect on gene activation or repression. Approximately 60% of all gene promoters include a CpG island (Li & Zhang, 2012). When the CpG island is unmethylated the promoter region is available for binding allowing for genes to be transcribed, when it becomes methylated the response is generally gene inactivation (Previti et al., 2009).

Within the last decade, however, there has been momentum in research to use DNA methylation for forensic tissue identification. The tissue source of a biological sample has been proven to affect the methylation level for a given sample. Therefore, the

main objective of this methylation-related research has been trying to find those CpG sites that are specific for the tissue-type in question. In order to do this, a selected CpG site must be either hypomethylated for one tissue type and hypermethylated for all others, or vice versa. There has been a large amount of success in uncovering blood-specific and semen-specific CpG sites (Lee et al., 2012; Madi et al., 2012), however, there have been very few vaginal epithelial-specific and saliva-specific CpG sites located.

Methylation Analysis Methods

The level of methylation at a particular CpG site can be found using several different methods such as ligation mediated PCR, restriction digestion PCR, and bisulfite modification followed by pyrosequencing. Ligation mediated uses enzymes that are methylation-sensitive to cut DNA sequences at restriction sites. The amount of enzyme activity is quantitated by PCR amplification of the cleaved DNA pieces, which directly correlates to the amount of methylation at the restriction site (McGrew & Rosenthal, 1993). For restriction digestion amplification a methylation-specific restriction enzyme is used to cleave restriction sites where methylation is not present. Tissue identification markers are amplified with the digested DNA and are fluorescently labeled. These PCR products are measured by capillary electrophoresis; the loci with higher methylation levels are going to amplify more efficiently and will give a greater signal (Frumkin et al., 2011).

A more recent method of methylation analysis can be accomplished by using bisulfite conversion techniques followed by pyrosequencing. The purpose of the bisulfite modification is to convert all unmethylated cytosine bases in a genomic sample to uracil while all the methylated cytosines remain as methylated cytosine (5mc). After the

modification, the DNA undergoes site-specific PCR amplification, followed by pyrosequencing. The DNA samples are denatured to single-stranded DNA and are exposed to a synthesized sequencing primer that anneals prior to samples entering the sequencing process (Delaney, Garg, & Yung, 2015). The single-stranded DNA is then synthesized, and with each incorporated nucleotide light is emitted and recorded. The amount of guanine or Adenine that is incorporated at a specific CpG site gives information about the amount of methylation present at that site in the sample being tested (Delaney, Garg, & Yung, 2015). If the same approximate level of methylation is seen at CpG sites across several samples, that methylation data can be compared to data for the same CpG site in other tissue samples. For the purposes of tissue discrimination, there needs to be a consistent trend of hypomethylation for one tissue while all others remain hypermethylated and vice versa. Pyrosequencing technology is advantageous for methylation analysis since it provides quantitative methylation data that can be used for tissue to tissue comparison.

Aims and Objectives

The main objective of this study is to locate a set of CpG sites that contain methylation levels consistent enough to discriminate saliva cells from other bio-fluids of forensic interest such as blood, vaginal epithelial tissue, and sperm. Ideally, if these CpG sites could be located for saliva, the data could be combined with methylation data for other tissue types to develop an assay capable of identifying the tissue source of an unknown DNA sample. Development of multiple markers for saliva identification will reduce the error rate in tissue identification. Since the DNA is already available for

forensic case work, there is no need to expend additional evidentiary item for this analysis.

CHAPTER II – REVIEW OF LITERATURE

Current Tissue Identification Methods

Currently, several presumptive and confirmatory tests exist as a whole for the identification of forensically-relevant body fluids. While these tests have become standard in many serological laboratories, they do have certain limitations. Many of these serological methods lack in either specificity or sensitivity, which can lead to false positive or false negative results.

Blood

One of the most common biological fluids found at crime scenes is blood. The presumptive tests for blood can be divided into two categories, namely catalytic color test and chemiluminescent test. The catalytic color test involves the change of color of the reagent used while chemiluminesence and fluorescence tests both emit light as the end product of an oxidation reaction in the presence of hemoglobin, a protein found in blood (Cassidy et al., 2017). Chemiluminescence does not require an alternate light source to be seen, however fluorescence tests do need an alternate light source to be visualized (Vandewoestyne et al., 2015) The most commonly used chemiluminescence test is Luminol, a presumptive test that has been found to have a sensitivity of detection for blood samples diluted up to 1:100,000 (Tobe et al., 2007).

The two most common catalytic color tests are Hemastix and Phenolphthalein. Both tests, as the name implies, produce a change in color that corresponds to the possible presence of blood in a sample. In a study conducted by Vennemann et al. (2014) the group was able to determine that the phenolphthalein presumptive test had a high sensitivity for neat blood samples at a 1:10,000,000 dilution, but showed poor results

when testing for specificity of blood detection. Hemastix test strips that were developed to detect occult blood in urine have found its way in forensic testing as well. A study conducted by Tobe et al. (2007) showed a sensitivity for Hemastix up to 1:100,000, but was unsuccessful in detecting blood at dilutions greater than this. The same group also tested the specificity of Hemastix using a number of various foods, chemicals, and other biological samples and found a large number of non-blood materials gave positive results for the test (Tobe et al., 2007). In addition, both catalytic color presumptive tests have been reported to cause DNA degradation (Thanakiatkrai et al., 2014). Since presumptive positive blood samples will be submitted for further DNA analysis, the DNA degradation should be kept to a minimum to the possible extent.

There are two serological tests that serve as confirmatory tests for the presence of blood: Takayama Crystal Test and the use of an ABAcard Hematrace test kit. The Takayama Crystal Test employs a Takayama reagent, which when added to a blood sample will produce pink colored feather-shaped crystals called pyridine ferroprotoporphyrin crystals that can be viewed under a microscope. This test is not specific to humans, and therefore cannot be used as a species-specific test, but only a confirmatory test. The most commonly used species-specific test for blood is the ABAcard Hematrace test card. This card is a one-step chromatographic test that relies on the specific reaction between human hemoglobin (Hb) antigens and anti-human hemoglobin antibodies (Hurley et al., 2009). The ABAcard Hematrace is described as having both high in specificity and sensitivity (Hurley et al., 2009), however it is not species specific. The ABAcard Hematrace test has shown positive blood detection results for human, primates, and ferrets (Johnston, Newman, & Frappier, 2003). The advantages

of this test do outweigh the disadvantages, as it is unlikely that the other species body fluids will be found at a crime scene.

Semen

Another biological sample of forensic interest is semen, generally present in cases where a sexual assault has taken place. Like other biological specimens, both a presumptive test and a confirmatory test are used to ascertain the presence of semen or spermatozoa. The presumptive test used for semen identification is called acid phosphatase, and is a color test that identifies acid phosphatase, which is found in the higher quantity in seminal fluid when compared to other secretions. In a study conducted by Gonçalves et al. (2017), the group tested the specificity of acid phosphatase presumptive test using semen and semen mixed with other biological fluids. It was found that The AP test was effective in giving a positive result in the presence of diluted semen samples, however was unsuccessful in discriminating between semen and other types of biological samples (Gonçalves et al., 2017). Another limitation of the acid phosphatase test is that the amount of acid phosphatase can vary largely between males (Redhead & Brown, 2013). It has also been found that the storage temperatures of the evidence affect the activity of acid phosphatase in the semen sample (Gaensslen 1983)

The two most commonly used confirmatory tests for semen identification are a microscopic search of spermatozoa and the detection of a prostate-specific antigen (PSA) using ABAcard p30 test strip. The identification of spermatozoa through a microscope has no specificity concerns since the spermatozoa are physically observed by the analyst. The only sensitivity concerns arise when there is a male individual who does not produce any spermatozoa, but this is a rare occurrence.

The ABAcard p30 test strip works for semen detection in the same way as the ABAcard Hematrace test strip works for blood detection, through antigen-antibody interactions. When a sample contains seminal material, the p30 antigen will bind with the anti-p30 antibodies, causing the formation of a colored band in the test kit. The previously mentioned study conducted by Gonçalves et al. (2017), also investigated specificity and sensitivity of the kit for p30 antigens and found that the kit was highly sensitive and specific for the p30 antigens, while other body fluids produced a negative result. (Gonçalves et al., 2017).

Vaginal Epithelial tissue

While there are several serological techniques available for the identification of blood and spermatozoa, there is no standard laboratory protocol exists for the identification of vaginal epithelial tissue. Earlier methods used for the identification of vaginal epithelial cells using glycogen staining were not reliable.

Saliva

Saliva contains an enzyme amylase that is present in higher quantities in saliva than other body fluids, making it a prime candidate for possible saliva detection. While the detection of saliva is neither a presumptive nor confirmatory test, the quantity of amylase in the sample can be used to make conclusions regarding the presence or absence of saliva in a sample. Amylase is a starch-hydrolyzing enzyme, used in conjunction with an iodine that can stain starch a blue color (Meyers & Adkins, 2008). These two factors are used together to determine the approximate quantity of amylase present in a sample. This information is then compared to known saliva, and non-saliva reference samples to determine the possible source of the unknown body fluid. One complication of using this

method is the interpretation of samples that are mixtures, which would have a varying concentration of amylase when compared to neat samples. Other tests such as the RSID-saliva identification kit determines the presence of the enzyme α-amylase in evidentiary samples (Casey and Price, 2010)

DNA Methylation Analysis

Compared to the progress that has been made for blood and semen identification, the literature regarding the identification of vaginal epithelial tissue and saliva are scanty. There have been several cases that support the validity of using methylation analysis for tissue identification. The first use of methylation analysis for forensic casework can be seen through the work of Frumkin et al. (2010) when the group developed an assay for the purpose of distinguishing artificial DNA from real DNA using methylation levels. The overall goal of such an assay was to increase the integrity of forensic DNA analysis, and as a result the group found that certain loci are consistently methylated where others are unmethylated. This fact opened the door to the possibility of distinguishing various forensically relevant tissue types using the same type of methylation analysis.

In 2011, Frumkin et al. published their work regarding the use of DNA methylation levels to distinguish between tissue types. The group used 50 samples including blood, semen, saliva, and skin cells that were first digested with a methylation-specific endonuclease, followed by amplification at specified regions and then analyzed using capillary electrophoresis. Using this methodology, the group was able to identify the source tissue for each sample tested using the methylation percentages obtained.

A study completed by Lee et al. (2011) further exemplified the ability to distinguish tissue types from one another using DNA methylation analysis. The study

investigated five differentially methylated regions (tDMR) of the DNA for the following sample types: saliva, blood, menstrual blood, vaginal fluid, and semen. Using bisulfite conversion methods, the group was able to determine that two of the markers in question were able to distinguish semen samples from all other tested sample types. Similarly, in a study conducted by Madi et al. (2012), four methylation markers were used to differentiate blood, saliva, and semen samples using pyrosequencing. Methylation data at these tDMRs are normally investigated using bisulfite modification of the genomic DNA, site specific PCR amplification and pyrosequencing. Madi et al were able to identify a panel of markers, C20orf117, ZC3H12D, BCAS4, and FGF7, that can be used in the determination of blood, saliva, semen and skin epithelial cells. Both of the studies presented by Lee et al. (2011) and Madi et al. (2012) show that investigating these tDMRs provides a way to locate markers that can be used for tissue identification.

In a broad study conducted by Park et al. (2014) the group was able to identify a large number of tDMRs using the Illumina HumanMethylation 450K bead array technology. Using this data, they were able to generate a list of the tDMRs for four different tissue types: saliva, blood, vaginal secretions, and semen. In addition to publishing these potential sites Park et al. (2014) also investigated two sites for each of the tissue type. They found that the eight selected sites possessed the ability to distinguish each of the tissue type in question.

It has become increasingly clear that DNA methylation can be used to distinguish tissue types from one another when the source of the sample is unknown. In a forensic point of view identifying the tissue source of a DNA sample is important because the presence or absence of a tissue may identify the type and severity of crime that took

place. Using the same bisulfite modification and pyrosequencing technology used by Madi et al. (2012), we hope to identify a set of novel saliva-specific CpG markers using potential candidate sites identified by Park et al. (2014).

CHAPTER III - MATERIALS AND METHODS

Sample Collection

Samples of four types of biological fluids, blood, semen, vaginal epithelial cells, and saliva were collected from volunteers under the conditions of an approved Institutional Review Board (IRB) protocol at The University of Southern Mississippi. All donor samples were assigned a unique identifier number to help maintain confidentiality and anonymity of the samples.

Blood samples were collected by first sterilizing the donor's fingertip with 70% ethanol, and then pricking the finger with a sterile autolet lancet device. A sterile cotton swab was used to absorb the blood sample, which was air dried, labeled with unique number, and stored frozen at -20°C.

The saliva samples were collected by swabbing the inside of the cheek of the volunteer for approximately 30 seconds with a sterile cotton swab. The sample was air dried, labeled with unique number, and stored frozen at -20°C.

For semen sample collection, a sterile sample collection cup was provided to the participants who collected the sample themselves in a manner that is private and convenient to them. The donor then provided the sample to the researcher, who stored the sample at -20°C freezer.

The vaginal epithelial samples were collected on a sterile cotton swab by the participant themselves in a manner that is convenient and comfortable to them. The sample was then returned to the researcher, labeled with a unique number, and stored in a -20°C freezer.

DNA Extraction

DNA was extracted using organic extraction techniques (Budowle, 2000). Cotton swabs containing samples of blood, saliva, or vaginal epithelial were separated from the applicator stick and placed in a labeled 1.5 mL tube. Each reaction contained 400 mL of stain extraction buffer, 10 microliters of Proteinase K, and was be incubated at 56°C overnight. After the incubation period is complete, the cotton swab was transferred to a new 1.5 mL tube containing a spin basket and centrifuged for five minutes at 12,000 rpm. The flow-through was placed back into the original 1.5 mL tube and the spin basket containing the cotton swab was discarded.

25 microliters of each semen sample was added to a tube that contained a modified stain extraction cocktail; 150 microliters TNE (Tris/NaCl/EDTA), 50 microliters of 20% Sarkosyl, 40 microliters of 0.39M dithiothreitol, 150 microliters of water, and 10 microliters of proteinase K solution and incubated at 37°C overnight.

Following incubation, 500 mL of phenol/chloroform/isoamyl alcohol was added to each sample and briefly vortexed until a milky emulsion is obtained. Samples were centrifuged for 10 minutes at 12,000 rpm to separate the aqueous and organic phases into two distinct layers. The aqueous phase was removed from each sample, placed into a concentrator, and then spun at 5,000 rpm for approximately ten minutes. Following this initial spin, the flow-through was discarded and ~0.5 mL TE was added to the concentrator. The samples were centrifuged for ten minutes at 5,000 rpm; the washing steps were repeated three additional times for a total of four washes. The filter unit was then inverted into a clean 1.5 mL tube and centrifuged at 3,000 rpm for approximately three minutes. This DNA was stored at -20 °C until further testing.

DNA Quantitation

A 1% agarose gels was used to determine both the quantity and quality of the extracted DNA. 1 μL of a 1Kb ladder was loaded into the well of each row in the agarose gel. 2 μL of each sample combined with 2 μL of Bromophenol Blue (1:1 diluted) was loaded into each well and electrophoresed in a 1x TAE buffer at 120V for twenty minutes. The gel was stained for approximately 5 minutes in ethidium bromide and the band intensities were photographed using a UV transilluminator. The band sizes and intensity were compared to the standard ladder to determine the quantity and quality of each DNA sample.

Bisulfite Conversion

Bisulfite conversions were performed using the recommended procedure provided with the Qiagen EpiTect Bisulfite Kit. Approximately 200 ng of genomic DNA were used for bisulfite conversion. The bisulfite conversation process is described below:

1. The components of each reaction for bisulfite conversion are listed in Table 1 below. Each component was added to a 0.2 mL tube

Component	**Volume per reaction (*μL*)**
DNA solution (1 ng-2 μg)	Variable* (maximum 20 *μL)*
Deionized water	Variable*
Bisulfite Mix solution	85
DNA Protect Buffer	35
Total volume	**140**

Table 1. Bisulfite conversion reaction components per sample

*The combined volume of DNA and water will total 20 microliters maximum

2. The samples were briefly vortexed, spun in the centrifuge, and placed in a thermalcycler.

Stage	Duration (minutes)	Temperature (°C)
Denaturation 1	5	95
Incubation 1	25	60
Denaturation 2	5	95
Incubation 2	85	60
Denaturation 3	5	95
Incubation 3	120	60
Hold	Indefinite	20

Table 2. Thermocycler conditions for bisulfite conversion.

3. The program for bisulfite conversion is described in Table 2.

Cleaning the Bisulfite Converted DNA

1. Each sample was removed from the thermalcycler and the contents were transferred to a clean 1.5 mL tube
2. 560 µL of loading buffer was added to the converted DNA, mixed and centrifuged gently.
3. Samples were transferred to a spin column that is provided with the EpiTect Bisulfite Kit and centrifuged at 12,000 rpm for 1 minute. The flow-through for this spin was discarded.
4. 500 µL of wash buffer was added to each tube. The samples were spun for one minute and the flow-through was discarded.
5. 500 microliters of desulfonation buffer was added to each tube, and incubated at room temperature for 15 minutes. The samples were spun at 12,000 rpm for one minute, and the flow through liquid was discarded.

6. 500 microliters of wash buffer was added to each sample and centrifuged for one minute at 12,000 rpm, the liquid from the spin will be discarded. This step was repeated a second time, providing the samples with two total washes with the wash buffer.
7. The columns were moved to new 2 mL tubes, centrifuged at 12,000 rpm for 1 minute, and then incubated with open lids for 5 minutes at 56°C to promote evaporation of the liquid.
8. The spin columns were placed in a new 1.5 mL tube and 20 µL of elution buffer was added directly onto the column. After one minute of incubation at room temperature, the samples were spun for one minute at 12,000 rpm. This step was repeated once more by adding another 20 µL elution buffer before centrifuging. The flow-through from these two spins contained the eluted bisulfite converted DNA.
9. The 1.5 mL tubes were labeled and placed in the -20°C freezer until further analysis.

Marker Selection and Primer Design

Park et al. (2014) has published the results of the Illumina 450k bead chip array data that contains potential CpG markers for multiple tissue identification. Using this data, several promising CpG sites for saliva identification were selected. The CpG site location in the human genome was determined and the information was entered into the University of California Santa Cruz (UCSC) genome browser. 200 base pairs of DNA sequence both at the 5' and 3' of the CpG site were selected and downloaded from the browser. This 400 base sequence was used to develop as assay for pyrosequencing using

the Pyromark assay design software (Qiagen Inc.) Ideally, the overall PCR product should be in the range of 100-200 base pairs.

Amplification of CpG Sites for Pyrosequencing

At least 10 samples of bisulfite converted DNA for each tissue type were selected for PCR amplification. A PCR master mix was created as described in Table 3.

Bisulfite-converted DNA	2 μL
10x PCR Primer set	2 μL
Coral load solution	2 μL
Q-solution	4 μL
2x Master Mix	10 μL

Table 3. PCR components and corresponding volume for amplification per sample.

Samples, along with a negative control, were placed in the thermocycler and a PCR amplification program was selected. This program is a cycling of three phases: denaturation, annealing of primer, and extension, and is described in Table 4.

Initial Incubation Step	Denaturation	Annealing	Extension	Final Extension	Final Soak
HOLD	**CYCLE (45 cycles)**			**HOLD**	**HOLD**
95ºC 15 min	94ºC 30 sec	Tm-5ºC 30 sec	72ºC 30 sec	72ºC 10 min	4ºC ∞

Table 4. Thermalcycler conditions for polymerase chain reaction amplification.

To determine the success of amplification, a 2% agarose gel was used. A 100 bp ladder was loaded in the leftmost well of each row followed by 2µl of samples, and was

electrophoresed at 120 volts for 20 minutes in a 1x TAE buffer. The gel was stained in ethidium bromide solution and a UV transilluminator was used to visualize and photograph the gel.

Pyrosequencing

To begin pyrosequencing, an assay was created using Pyromark Q24 software. The same software was used to determine the amount of enzyme, substrate, and dNTPs to be added to the cartridge for the sequencing reaction.

A cocktail containing 2 microliters of streptavidin beads, 20 microliters of water, and 40 microliters of binding buffer per sample was made. 62 microliters of cocktail was added to each well of a 24-well plate, along with 18 microliters of PCR product. The sample wells were covered with a strip cap and shaken at 1000 rpm for 10 minutes. The 24-well plate was removed from the shaker and the samples were processed using a vacuum workstation. The vacuum work station was used to lift the samples, washed with 70% ethanol, denatured in NaOH solution and neutralized. After processing, the samples were released into a pyrosequencing plate that contains 25 microliters of 1x sequencing primer in each well. The pyrosequencing plate was incubated for two minutes at 80°C, followed by 10 minutes at room temperature for annealing of the sequencing primers to the target.

The pyrosequencing cartridge was prepared according to the quantities determined in the pre-run information from the Pyromark Q24 software. Following the addition of all materials to the pyrosequencer, the samples were ready to be sequenced.

Pyrosequencing Data Analysis

Methylation data from the pyrosequencer was presented as a pyrogram, showing relative methylation percentages at each CpG site within the target region. This data was imported to an excel workbook where preliminary analysis was conducted regarding the average percent methylation at each site as well as the standard deviation. A histogram was created for each marker that includes the data from all four tissue types. Those sites that look promising for saliva differentiation were analyzed further using SPSS statistical analysis package software for the level of significance of the methylation data from different tissues. A one-way ANOVA was used to compare the mean methylation levels of different tissues. A p value of 0.05 or below ($p<0.05$) was considered significant.

Species Specificity Test

The species specificity study involves testing of non-human samples using PCR and pyrosequencing. Several different species were tested using marker cg-9652652. The samples tested were cat, dog, chicken, cow, erythrobacter, chimp, rhesus, and a human control buccal sample. All genomic DNA samples were quantitated either by agarose gel or by human DNA quantifiler kit (Applied Biosystems), bisulfate modified and PCR amplified using the primers for the marker cg-9652652. The robustness of the amplification was tested using a 2% agarose gel. All samples were sequenced by pyrosequencing and the data was analyzed to determine if any species showed quantifiable methylation data

Mixture study

For the mixture study one semen sample and one saliva sample were used. The saliva sample had hypermethylation and semen sample was hypomethylated. The two samples

chosen were based on success of previous PCR amplifications. Each sample was diluted to a concentration of 10 ng/µl using TE buffer. Five different ratios of saliva v/s semen were set up, which include 90-10, 72-25, 50-50, 25-75 and 10-90. Each ratio contained a total combined amount of 100 ng of DNA, and was conducted in duplicate. After the mixture was created, the samples were bisulfite modified followed by and PCR amplification. A 2% gel was used to determine the success of the PCR amplification followed by pyrosequencing. The data for these mixtures were averaged and analyzed for any significant patterns.

CHAPTER IV – RESULTS

The genomic DNA quantitation using agarose gel was performed to assess the quantity and quality of the DNA extracted from all tissues followed by bisulfate conversion, PCR and pyrosequencing. Ten or more samples per tissue type were used for pyrosequencing. The methylation analysis includes the average methylation of each CpG sites for each tissue types.

Marker cg-9652652

For the marker cg-9652652, ten or more samples per tissue type were used for methylation analysis. The methylation percentages for all CpG sites were analyzed using excel program. The average and standard deviation data are shown in table 5.

	CpG_1	CpG_2	CpG_3	CpG_4	CpG_5	CpG_6
Buccal	61.0 ±14.6	52.4 ± 11.5	53.9 ± 55.1	55.1 ± 13.9	49.3 ± 8.9	60.3 ± 15.9
Blood	14.8 ± 14.5	12.5 ± 11.6	12.5 ± 12.4	14.4 ± 15.1	11.1 ± 10.4	13.7 ± 14.3
V. Epi	12.5 ± 13	10.0 ± 10.7	9.5 ± 11.0	10.8 ± 11.8	10.5 ± 10.3	11.0 ± 10.7
Sperm	2.7 ± 2.2	2.3 ± 1.7	2.0 ± 1.6	2.3 ± 1.9	3.5 ± 3.1	2.3 ± 1.7

Table 5. Percent Methylation and Standard Deviation for cg-9652652

The same methylation data are shown as a histogram in figure 1.

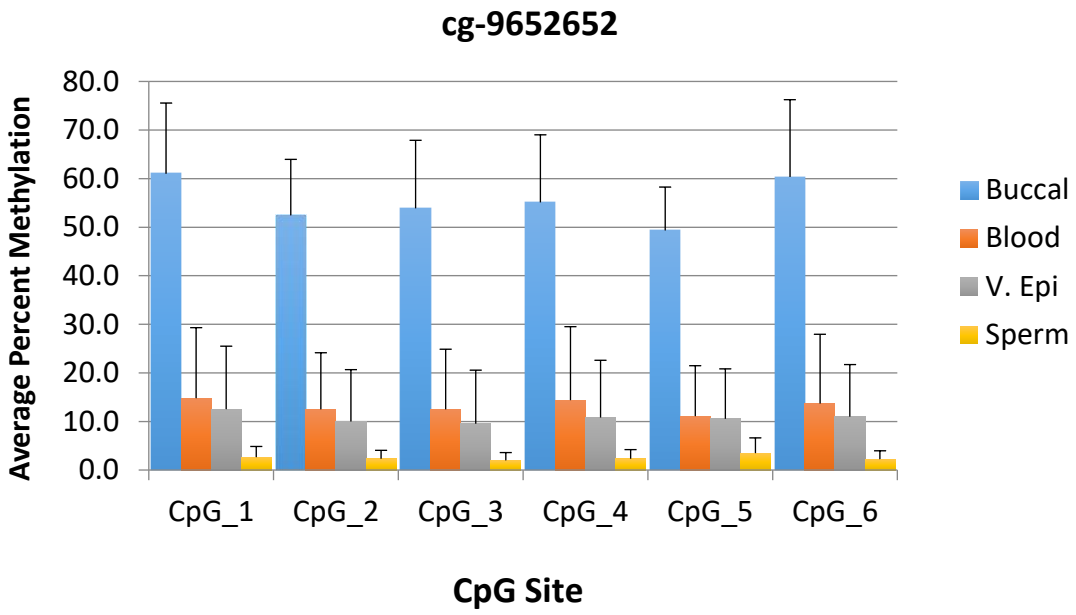

Figure 1. Histogram of average methylation data of marker cg-9652652. n=12 buccal; 10 blood; 11 v.epi; 12 sperm.

The level of significance ($p<0.05$) in the methylation data between different tissues were calculated using SPSS statistical package with one-way ANOVA and tukey's posthoc parameters. It was found that the methylation data for all CpG sites were significantly different ($p = 0$) compared to other tissues.

Marker cg-11536474

The average methylation data for the marker cg-11536474 for the four tissues studied are shown in table 6.

	CpG_1	CpG_2	CpG_3
Buccal	76.6 ± 18.9	72.8 ± 20.9	69.2 ± 20.1
Blood	10.4 ± 8.4	10.5 ± 12.1	8.1 ± 8.5
V. Epi	10.1 ± 3.6	12.2 ± 4.3	8.5 ± 2.9
Sperm	7.3 ± 8.2	8.0 ± 5.8	3.2 ± 2.3

Table 6. Percent Methylation and Standard Deviation for cg-11536474

Figure 2 shows the methylation data for the four tissues for marker cg-11536474.

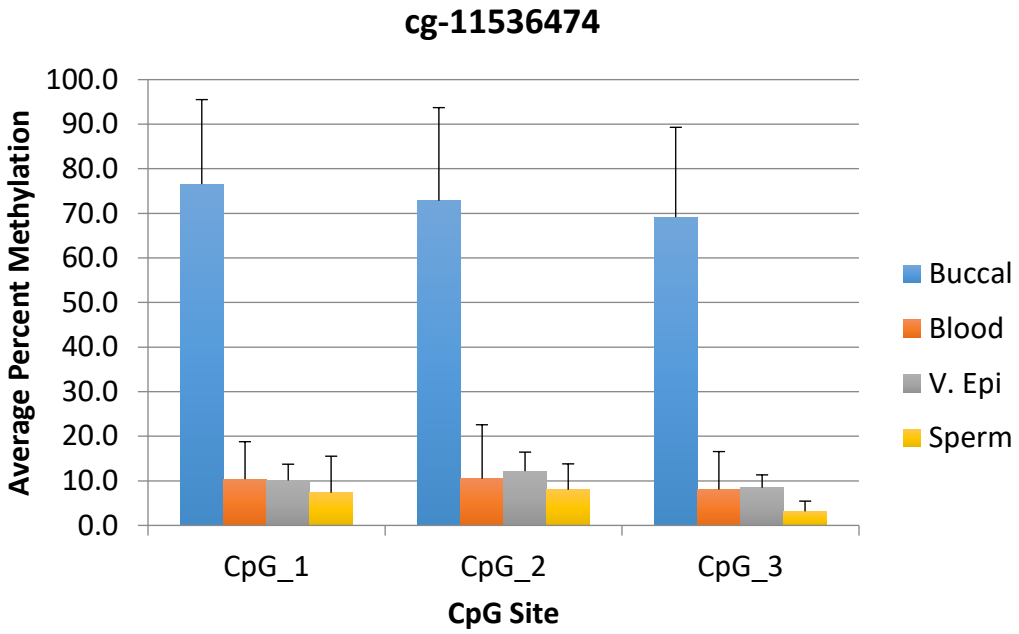

Figure 2. Histogram of the average methylation data of marker cg-11536474. n=12 buccal; 18 blood; 11 v.epi; 12 sperm.

It was found that the methylation data for all CpG sites were significantly different ($p = 0$) compared to other tissues. This data suggests that the marker cg-11536474 can be used to differentiate saliva from other tissues in forensic tissue identification.

Marker cg-3867465

The average methylation data for the marker cg-3867465 for the four tissues studied are shown in table 7.

	CpG_1	CpG_2	CpG_3	CpG_4	CpG_5
Buccal	47.6 ± 11	54.4 ± 13	74.5 ± 15.7	72.4 ± 14.7	68.4 ± 14.6
Blood	4.1 ± 2.2	5.6 ± 2.6	10.1 ± 5	9.8 ± 5.2	6.0 ± 1.7
V. Epi	3.6 ± 1.5	5.3 ± 4.1	9.5 ± 7.2	10.1 ± 5.3	9.6 ± 4.7
Sperm	2.1 ± 0.8	2.2 ± 1.1	3.9 ± 1.7	5.6 ± 2.9	6.5 ± 3.3

Table 7. Percent Methylation and Standard Deviation for data for cg-3867465

Figure 3 shows the methylation data for the four tissues for marker cg-3867465.

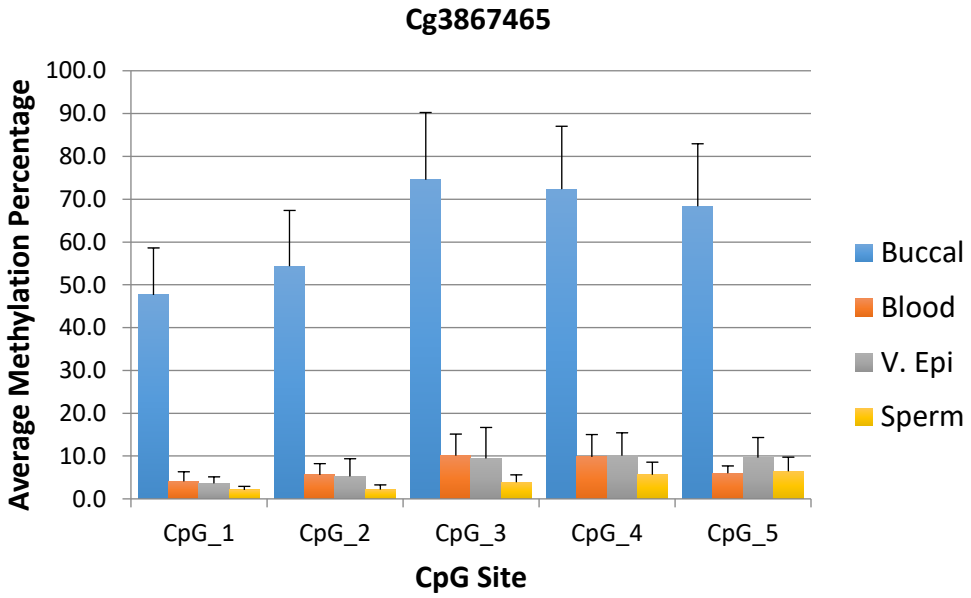

Figure 3. Histogram of average methylation data of marker cg-3867465. n=11 buccal; 10 blood; 11 v.epi; 11 sperm.

It was found that the methylation data for all CpG sites were significantly different ($p = 0$) compared to other tissues. This data suggests that the marker cg-3867465 can be used to differentiate saliva from other tissues in forensic tissue identification.

Marker cg-10781408

The average methylation data for the marker cg-10781408 for the four tissues studied are shown in tables 8.1 and 8.2.

	CpG_1	**CpG_2**	**CpG_3**	**CpG_4**	**CpG_5**	**CpG_6**
Buccal	72.0 ± 18.9	75 ± 19	68 ± 20.2	77 ± 19.8	52.5 ± 17.6	56.0 ± 17.5
Blood	3.4 ± 2.5	2.3 ± 1.8	2.3 ± 2	2.5 ± 0.8	4 ± 1.8	1 ± 0.7
V. Epi	16 ± 6.7	15.2 ± 6.7	12.6 ± 6	18.4 ± 7.4	16.8 ± 5.7	11.3 ± 5.1
Sperm	5.8 ± 4.5	4.3 ± 2.2	3.9 ± 3.3	4.8 ± 3.2	15.3 ± 9.6	3.8 ± 2.1

Table 8.1. Percent Methylation and Standard Deviation for cg-10781408 sites 1-6

	CpG_7	**CpG_8**	**CpG_9**	**CpG_10**	**CpG_11**
Buccal	52 ± 16.2	56 ± 16.9	52 ± 16.6	60 ± 18.4	62.0 ± 18.7
Blood	3 ± 1.7	2.5 ± 0.8	7.0 ± 3.1	4.0 ± 1.4	3.0 ± 1.1
V. Epi	12 ± 5.1	12.5 ± 5.4	14.6 ± 6.3	12.6 ± 6.4	14.5 ± 6
Sperm	5.6 ± 3.4	7.5 ± 2.7	8.0± 3.4	6.5 ± 3.9	6.5 ± 3

Table 8.2. Percent Methylation and Standard Deviation for cg-10781408 sites 7-11

Figure 4 shows the methylation data for the four tissues for marker cg-10781408.

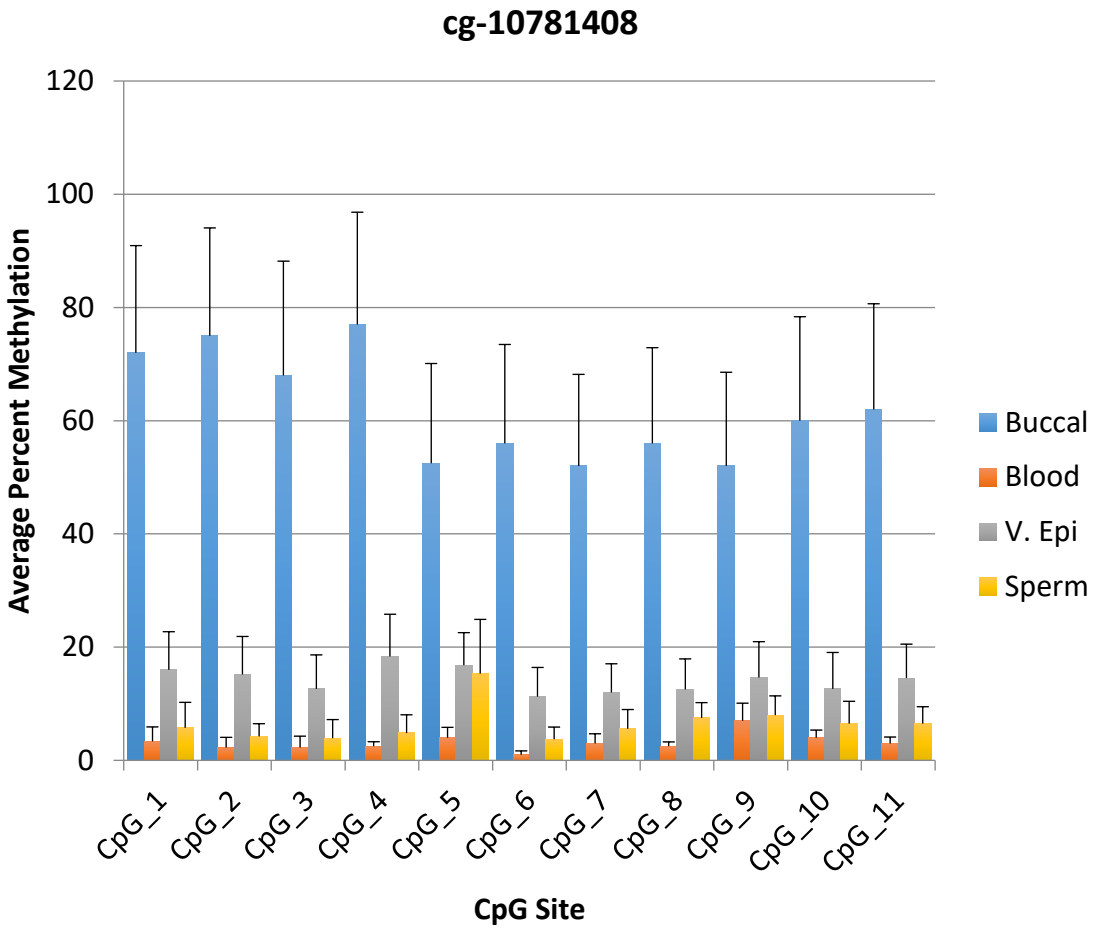

Figure 4. Histogram of average methylation data of marker cg-3867465. n=12 buccal; 11 blood; 11 v.epi; 12 sperm.

It was found that the methylation data for all CpG sites were significantly different ($p = 0$) compared to other tissues. This data suggests that the marker cg-10781408 can be used to differentiate saliva from other tissues in forensic tissue identification.

Marker cg-10122865

The average methylation data for the marker cg-10122865 for the four tissues studied are shown in table 9.

	CpG_1	CpG_2	CpG_3	CpG_4	CpG_5
Buccal	85.6 ± 21.4	65.9 ± 18.5	73.4 ± 18.8	58.7 ± 17.2	75.1 ± 21.4
Blood	7.2 ± 2.4	5.2 ± 1.6	2.6 ± 1.9	3.5 ± 4.1	5.8 ± 3.2
V. Epi	15.4 ± 7.8	11.3 ± 6	9.8 ± 8.3	8.1 ± 5.8	13.7 ± 6.1
Sperm	7.0 ± 4.4	12.4 ± 9	3.4 ± 2.3	4.5 ± 4	13.3 ± 7.9

Table 9. Percent Methylation and Standard Deviation for cg-10122865

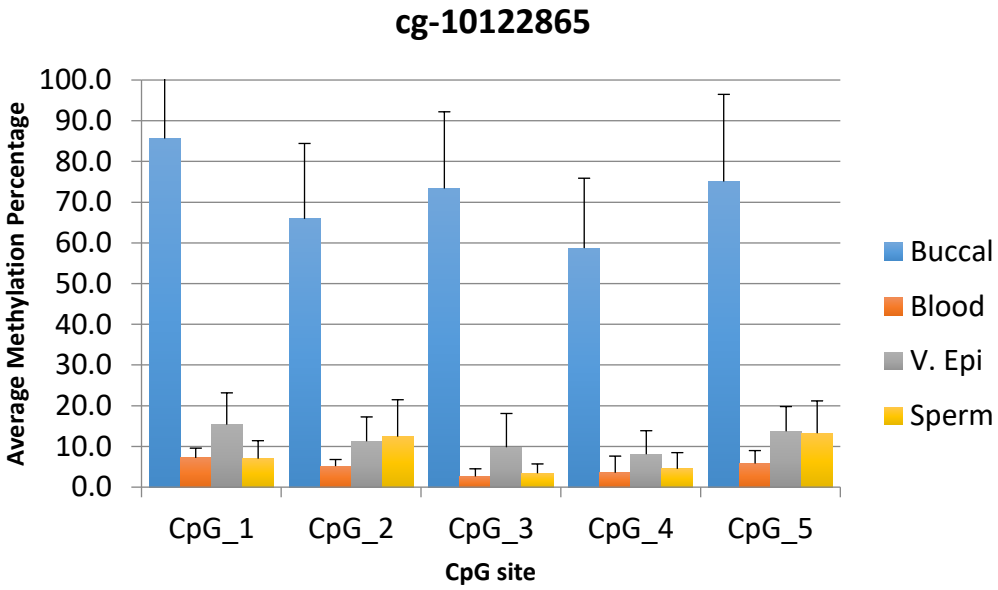

Figure 5. Histogram of average methylation data of marker cg-10122865. n=11 buccal; 11 blood; 11 v.epi; 12 sperm.

It was found that the methylation data for all CpG sites were significantly different ($p = 0$) compared to other tissues. This data suggests that the marker cg-10122865 can be used to differentiate saliva from other tissues in forensic tissue identification.

Species Test

In order to know the specificity of the primers to see if any other species will react with the human assay, several other organisms were tested. For this purpose, the following samples were tested: cat, dog, chicken, cow, erythrobacter, chimp, and rhesus. When amplified for the marker cg9652652, only the chimp, and rhesus samples amplified that was detected with a 2% agarose gel. All samples used in the amplification were sequenced regardless of amplification success. Tables 10.1 and 10.2 show the methylation data obtained from different species.

	CpG_1	**CpG_2**	**CpG_3**	**CpG_4**	**CpG_5**
Chimp	6	2	2	3	1
Rhesus	85	76	82	94	84
BUC 147	88	75	73	80	59

Table 10.1 Methylation data obtained for the different species, sites 1-5

	CpG_6	**CpG_7**	**CpG_8**	**CpG_9**
Chimp	2	7	7	2
Rhesus	3	3	5	95
BUC 147	77	58	78	84

Table 10.2 Methylation data obtained for different species, sites 6-9

The chimp sample was hypermethylated in all CpG sites tested while the rhesus sample produced mixed results, with CpG sites 1-5 and 9 being hypermethylated, and site 6-8 hypomethylated. All other species tested did not produce any methylation data. These results show that the primers are human specific and can be used to identify human tissues in a forensic scenario.

Mixture Study

Often the samples submitted for forensic analysis are mixtures of two different individuals or samples of two different tissues. To study how a sample with two different body fluids mixed will behave in pyrosequencing, mixtures of two different body fluid DNA were prepared in different ratios (buccal versus sperm %): 90-10, 72-25, 50-50, 25-75, and 10-90. All samples showed amplification bands of the right size and were pyrosequenced. The methylation data of the different ratios for CpG site 2 in marker cg-9652652 is shown in Figure 6. As expected, the methylation increased when the saliva sample is predominant and decreased when the sperm sample is predominant.

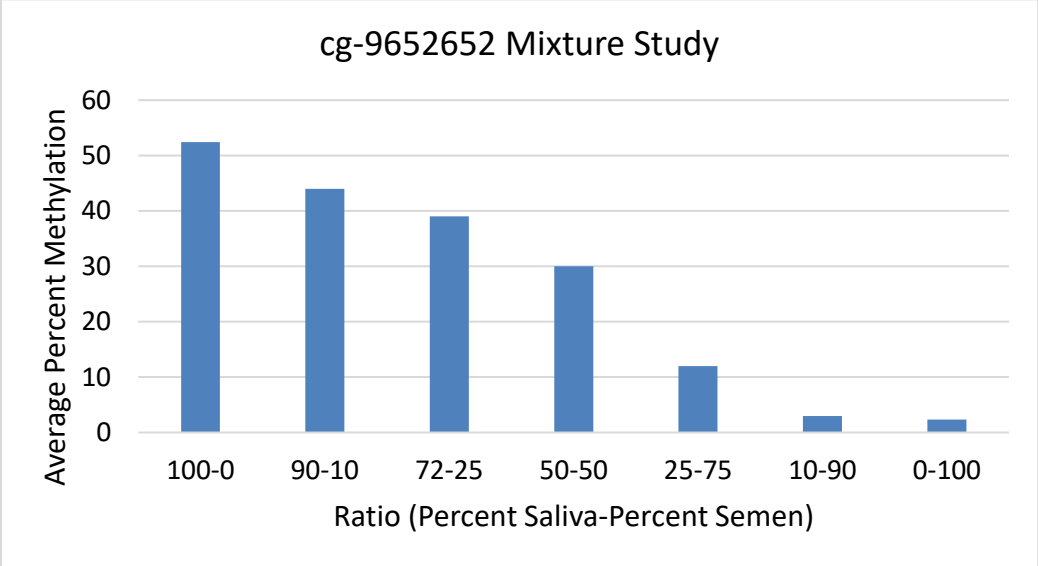

Figure 6. Methylation percent averages of mixed samples for cg-9652652, CpG site 2.

CHAPTER V – DISCUSSION

Tissue identification in a forensic setting may provide vital information about the type and severity of a crime that has taken place. Coupled with DNA analysis, information could be gained that may determine the individual(s) who were present at the scene. The use of methylation analysis has received more attention in the recent decade as an alternate option for tissue identification (Frumkin et al., 2010; Frumkin et al., 2011; Lee et al., 2012; Madi et al., 2012; Park et al., 2014). DNA Methylation is an epigenetic process that can alter the gene function without changing the underlying DNA sequence itself. In previous years, DNA methylation has been studied for medicinal purposes, as methylation site proximity to the promotor region of genes control gene regulation. (Li & Zhang, 2012; Previti et al., 2009). It has been determined that using bisulfite conversion followed by PCR and pyrosequencing can provide quantitative methylation levels of a CpG site. Pyrosequencing is a process that provides a ratio of converted to unconverted cytosine bases in a CpG site to determine the percent methylation of that site in question. The same analytical technique has been applied in the identification of four forensically-relevant tissue types: blood, semen, saliva, and vaginal epithelial tissue. Lee et al. (2012) and Madi et al. (2012) have used this pyrosequencing technology to study the methylation data for the identification of different tissues. In spite of the success of this technology, data regarding the identification of other tissues such as vaginal epithelial tissue and saliva are rare.

Therefore, this present study was undertaken to determine the usefulness of methylation data in differentiating saliva from three other forensically relevant tissues such as blood, semen and vaginal epithelia. In a study conducted by Park et al., (2014),

the investigators located a large number of potential CpG sites that can differentiate saliva from other tissues using Illumina 450K bed chip array technology. This preliminary data was used to study certain saliva specific markers that may be used for the differentiation of saliva from other tissues.

Saliva identification:

Several potential CpG sites mentioned by Park et al (2014) were screened and five methylation markers were identified which showed promise in the differentiation of saliva from the other tissues. The identified markers were cg9652652, cg11536474, cg3867465, cg10781408, and cg10122865 along with additional CpG sites that flank these sites.

All the five markers mentioned above showed hypermethylation in saliva while the other tissues were hypomethylated. The percent methylation levels between saliva and all other tissue types were found to be statistically significant ($p<0.05$) for all five markers tested, giving the conclusion that these five markers could be used to differentiate saliva from blood, semen, and vaginal epithelial samples.

Species specificity of the markers:

In a forensic setup, identification of the species of origin of a tissue sample is important since the unknown crime scene samples may contain non-human DNA. To determine if the assay developed for the markers are human specific, one of the markers studied (cg9652652) was chosen to determine if non-human samples will amplify and provide any methylation data. Several different species were tested including cat, dog, chicken, cow, erthyrobacter, chimp, rhesus, and a human control. All samples were analyzed using pyrosequencing and only the chimp and rhesus samples gave usable

methylation data. It is not unexpected that the two primate samples amplified with the human specific primers because of the high similarity of the primate genome with that of humans. All other non-primate samples did not produce any methylation data and this proves that the PCR primers used in this study are human specific and will not interfere with non-human DNA samples.

Mixture studies:

It is often possible that the DNA samples recovered from crime scenes are either mixture of two different individuals or of two different cell types. In order to study how a sample with two different cell type mixture behaves in their methylation content, a controlled mixture of two different cell types and of different ratios were studied. A sperm sample that was hypomethylated and a saliva sample that was hypermethylated were used. As theoretically expected, when the quantity of saliva was reduced, the methylation percent of the mixture also was reduced. These mixture studies help in deciphering of different components and quantity of each tissue type.

In addition to tissue identification, other studies have attempted to correlate the age of an individual and methylation (Weidner et al., 2014; Park et al., 2016; Alghanim et al., 2017). Some studies have reported the success in differentiating different age group individuals with methylation data (Jenkins et al., 2014, Vidaki et al., 2016). The ability to estimate age in a forensic setting when the source of a tissue sample is unknown would have significant implications.

Using methylation analysis for tissue identification over traditional serological tests has several advantages. First, traditional serological tests are used presumptively to determine if additional testing is warranted. Additional tests can lead to the consumption

of evidentiary material. For methylation analysis, the analyst does not have to extract the DNA separately because the DNA is already available for case work analysis.

Most traditional serological tests rely on the specific interaction of proteins within the tissue in question and the chemicals used for testing. The second advantage of methylation analysis for tissue differentiation is that this test relies on DNA, which is more stable that the protein used in serological analysis, and the proteins can degrade over a period of time. Third, there is also a potential for multiplex kit development where more than one marker can be analyzed simultaneously either for tissue identification or age determination or a combination of both. This would reduce the cost associated with individual marker analysis.

Limitations of this study include the influence of external factors such as age, smoking, diet, and obesity. It has been reported that factors such as smoking and obesity contribute to the methylation status of an individual (Vidaki et al., 2013). So, it should be kept in mind that external factors can contribute to the differences in methylation among the experimental population.

In conclusion, it is determined that the five markers identified in this study have the potential to differentiate saliva from other forensically relevant tissues. Also, the primers used in the assay appear to be human specific with no interference of non-primate samples.

APPENDIX A – Primer Specifications

Marker	Primer Sequence	PCR Product Size
Cg9652652	Forward: GAGTTTTATTAGGGTTGAGTTTTT Reverse: CCCCAAATACCCCATTTCC Sequencing: AGTTTTATTAGGGTTGAGTTTTTT	125 bp
Cg11526474	Forward: GAGTTAGGTTGTAGTAAAGTTT Reverse: ACTACCCCCCTATAAACCTCTAC Sequencing: GAGTTAGGTTGTAGTAAAGTTTT	129 bp
Cg3867465	Forward TTTGGAGAGTTGAGTATTTGTGTGGTAAG Reverse: ACCTCTAACCCCTCTCAACAAACTCTAC Sequence: TGGTAAGAGGGGTTT	150 bp
Cg10781408	Forward: GTAGTATAGGAAGTTTAGGTGGAAGA Reverse: ATCCAAACCTCACTCTCTATCC Sequencing: AGTTTAGGTGGAAGAG	118 bp
Cg10122865	Forward: GGGGGTTAGGAGAGTTTAAGA Reverse: ACACCAAACCACCTTTTCT Sequencing: AGTTTAAGAAGTGGGG	111 bp

APPENDIX B –IRB Approval Letter

Office *of*
Research Integrity

THE UNIVERSITY OF SOUTHERN MISSISSIPPI

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Modification Institutional Review Board Approval

The University of Southern Mississippi's Office of Research Integrity has received the notice of your modification for your submission DNA Methylation Analysis for Tissue-Type and Age Determination for Forensic Use (IRB #: IRB-19-68).

Your modification has been reviewed by The University of Southern Mississippi Institutional Review Board in accordance with Federal Drug Administration regulations (21 CFR 26, 111), Department of Health and Human Services regulations (45 CFR Part 46), and University Policy to ensure:

- The risks to subjects are minimized and reasonable in relation to the anticipated benefits.
- The selection of subjects is equitable.
- Informed consent is adequate and appropriately documented.
- Where appropriate, the research plan makes adequate provisions for monitoring the data collected to ensure the safety of the subjects.
- Where appropriate, there are adequate provisions to protect the privacy of subjects and to maintain the confidentiality of all data.
- Appropriate additional safeguards have been included to protect vulnerable subjects.
- Any unanticipated, serious, or continuing problems encountered involving risks to subjects must be reported immediately. Problems should be reported to ORI via the Incident template on Cayuse IRB.
- The period of approval is twelve months. An application for renewal must be submitted for projects exceeding twelve months.

PROTOCOL NUMBER: IRB-19-68
PROJECT TITLE: DNA Methylation Analysis for Tissue-Type and Age Determination for Forensic Use
SCHOOL/PROGRAM: School of CJFS, Criminal Justice, Forensic Sci
RESEARCHER(S): Elizabeth Staples ,Kuppareddi Balamurugan

IRB COMMITTEE ACTION: Approved

2b. Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

PERIOD OF APPROVAL: October 30, 2019

Donald Sacco Jr.

Donald Sacco, Ph.D.
Institutional Review Board Chairperson

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